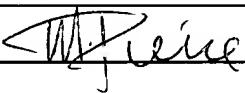


EXPRESS MAIL CERTIFICATE			
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.			
Typed or Printed Name	Margaret D. Pierce	Express Mail No.	EL 563 388 639 US
Signature		Date	March 19, 2001

INTEGRIN BINDING MOTIF CONTAINING PEPTIDES AND METHODS OF TREATING SKELETAL DISEASES

CROSS-REFERENCE

[0001] This application is a continuation-in-part application of Serial No. 09/641,034, filed August 16, 2000, which is incorporated herein by reference in its entirety and to which application we claim priority under 35 USC § 120.

FIELD OF THE INVENTION

[0002] The invention relates generally to the field of peptides and more particularly to peptides and formulations thereof useful in treating skeletal diseases.

BACKGROUND OF THE INVENTION

[0003] It is well-documented that disorders of skeletal tissues and mineral metabolism cause numerous significant health problems on world-wide basis.

[0004] In humans, the maximum bone mass occurs between the age of 15 and 40 and is referred to as "peak bone mass." After such peak bone mass age, bone mass begins declining gradually and the mechanical strength of the bone is accordingly reduced. Consequently, when mechanical strength declines to a certain level, the individual is at greater risk of bone fracture. This natural occurrence is called osteoporosis if severe enough to be pathogenic.

[0005] The speed at which bone loss occurs differs among individuals, and especially with respect to gender. In females, the speed of bone loss accelerates immediately after menopause (See Figure 1) because of a significant decline in available estrogen, a hormone which plays a critical role in maintaining healthy bone metabolism. Postmenopausal osteoporosis constitutes an important clinical problem because it afflicts

significant numbers of women. Notably, the ratio of female to male osteoporosis patients is 3:1.

[0006] The majority of bone diseases are characterized by loss of bone minerals, weakening of bones and consequently, an increase of the frequency and severity of bone fractures, which are called "pathological fracture." In the elderly population, this has significant social ramifications as well, as many of those with bone fractures have difficulty with mobility, which often leads to the deterioration of other mental and physical functions, resulting in dementia, muscular weakness and/or fatigue. In addition, morbidity and pain are significantly increased by thrombotic events, such as pulmonary embolism which can occur as a result of hip or pelvic fractures.

[0007] In the United States alone, it is said that 52 million women over age of 45 will suffer from osteoporosis by 2000. Current worldwide osteoporosis population is around 200 million. Annual incidence of pathological fracture in the United States alone is approximately 1.5 million. It is estimated that annual medical costs for those osteoporosis patients in the United States and world are \$14 billion and \$60 billion, respectively.

[0008] Renal failure is also a significant health problem related to mineral metabolism and skeletal formation, and the number of its patients is increasing rapidly. Renal function is declining gradually over several to ten years period in these patients. When the renal function becomes approximately a quarter (1/4) of the healthy level, the patients are classified to chronic renal failure. When it becomes approximately one sixth (1/6) thereof, they need to start dialysis and are called end stage renal disease (ESRD). In patients with chronic renal failure, serum levels of important minerals such as calcium and phosphate lose their normal homeostasis, which results in malformation of skeleton. It is called renal osteodystrophy (ROD), which is a secondary osteoporosis from renal failure. ROD can also cause pathological fracture like osteoporosis. The prevalence of end stage renal disease (ESRD) in the United States is rapidly increasing and about to reach 300 thousand in 2000. ROD affects most ESRD patients.

[0009] There are several other diseases of skeletal tissues and mineral metabolism such as Paget's Disease, rickets, osteopetrosis, hyperparathyroidism, and so forth and a number of patients are affected by these diseases.

- [0010] Metabolically, bone is a highly active organ with bone resorption and formation occurring continuously (remodeling). Bone resorption is facilitated by osteoclasts which are differentiated from monocyte/macrophage lineage cells. Osteoclasts adhere to the surface of bone and degrade bone tissue by secreting acids and enzymes. Osteoblasts facilitate bone formation by adhering to degraded bone tissue and secreting bone matrix proteins, which are mineralized mostly by calcium and phosphate. Osteoblasts differentiate into bone cells (osteocytes), and become a part of bone tissue.
- [0011] Numerous experimental approaches have been attempted to either accelerate bone formation or diminish bone resorption. For example, growth factors such as BMPs (bone morphogenetic proteins), TGF β (transforming growth factor β), IGF (insulin-like growth factor), and fibroblast growth factor (FGF) are known to have potent biological activities in bone formation. In particular, a few subfamily molecules of BMP such as BMP-2 is regarded as one of the most potent growth factors for hard tissue. However, these factors have not been developed as therapeutic agents for systemic bone diseases. It is because none of them can be delivered to the bone selectively and some of these factors such as BMPs convert soft tissue into hard tissue. It is called ectopic calcification and is a critical adverse effect for them when they are used systemically. Further, the processes of bone formation and resorption are so closely connected and that makes selective increase of bone formation or selective inhibition of bone resorption extremely difficult.
- [0012] Currently, there is a need for an effective treatment for bone loss. Therapeutic agents such as estrogen, calcitonin, vitamin D, fluoride, Ipriflavon, bisphosphonates, and a few others have failed to provide a satisfactory means of treatment. (Gennari et al., *Drug Saf.* (1994) 11(3):179-95).
- [0013] Estrogen and its analogues are frequently administered to patients with postmenopausal osteoporosis. Estrogen replacement therapy involves administration of estrogen just prior to or after the onset of menopause. However, as is often the case with steroid hormones, the long term use of estrogen has significant adverse effects such as breast and other gynecological cancers (Schneider et al., *Int. J. Fertil. Menopausal Study* (1995) 40(1):40-53).
- [0014] Calcitonin, an endogenous hormone produced by the thyroid, binds selectively to osteoclasts, via its receptor, and inactivates them. Since the osteoclast is the only cell

which can dissolve bone tissue, calcitonin binding can block or slow down bone degradation caused by the osteoclast. However, this biological mechanism is very short-lived, as the osteoclasts become tolerant to this drug relatively quickly. Therefore, the use of calcitonin does not provide an effective therapeutic option.

- [0015] Fluoride has been shown to increase bone mass when it is administered to humans. However, while bone mass is increased, mechanical strength is not. Therefore, despite the increase in apparent bone mass, the risk of fracture remains (Fratzl et al., J. Bone Mineral Res. (1994) 9(10):1541-1549). In addition, fluoride administration has significant health risks.
- [0016] Ipriflavon has been used to treat osteoporosis in limited areas in the world. However, the actual efficacy of this compound is questionable and it is not widely accepted as a useful therapeutic agent for bone diseases.
- [0017] Bisphosphonates are compounds derivatized from pyrophosphate. Synthesis involves replacing an oxygen atom situated between two phosphorus atoms with carbon and modifying the carbon with various substituents. While bisphosphonates are known to suppress bone resorption, they have little effect on bone formation. Furthermore, bisphosphonates adhere to the bone surface and remain there for very long time causing a long-term decrease in bone tissue turnover. As bone tissue needs to be turned over continuously, this decrease in turnover ultimately results in bone deterioration (Lufkin et al., Osteoporos. Int. (1994) 4(6):320-322; Chapparel et al., J. Bone Miner. Res. (1995) 10(1):112-118).
- [0018] Another significant problem with the agents described above is that with the exception of fluoride and ipriflavon, they are unsuitable for oral administration, and thus, must be given parenterally. Since bone disorders are often chronic and require long-term therapy, it is desirable that therapeutic agents be suitable for oral administration.
- [0019] In summary, a significant need exists for a therapeutic agent which can prevent or treat bone loss. In particular, a new drug that can selectively increase bone formation and/or number of osteoblast without affecting bone resorption or soft tissue is highly desired.
- [0020] Another major health problem relating to skeleton and mineral metabolism is that with teeth. In the United States alone, it is estimated that 67 million people are affected by periodontal disease and that the annual cost of its treatment is approximately \$6.0

billion in 2000. It is said 90% of the entire population experience dental caries in their lives. The annual cost to treat them is over \$50 billion per year in the United States alone.

[0021] Dental caries are a universal disease and affects children and adults. Periodontal disease, on the other hand, affects mostly adults, and in particular, the aged. In many cases, the patient's gum is inflamed and destroyed, and the alveolar bone that supports the teeth is deteriorated. Cement that composes the core of the root is also damaged, and subsequently, teeth fall out. One of the most common treatments for tooth loss involves the use of a dental implant. An artificial implant (osseointegrated dental implants) is placed in the space where the tooth was lost. In severe cases, an entire denture is replaced by implants. However, implants frequently loosen, or fall out because their fixation on the alveolar bone is not always successful. Since alveolar bone is somehow damaged in these patients, the implant cannot always be supported well by alveolar bone. When alveolar bone is severely damaged, autogenous bone grafting is performed. In this case, a bone graft taken from another skeletal tissue of the same patient is grafted in the damaged alveolar area so that the hard tissue is regenerated and sinus is elevated there. Since these treatments require expensive bio-compatible materials and/or highly skilled techniques, the cost of treatment is usually very high.

[0022] It is believed that dental caries are caused by acidic condition in the oral cavity. For instance, sugars are converted to acid and dissolve the surface of the teeth. Although only enamel and a part of dentin is affected in many cases, the damage can reach the pulp cavity in severe cases that cause significant pain. The most typical treatment is filling the caries lesion with non-degradable materials such as metals or metal oxide. Treatment of dental caries mostly depends upon those materials and the techniques by the dentists, which is often expensive.

[0023] Although a few therapeutic agents have been developed and used in dental area, they are generally only anti-inflammatory drugs, analgesics, and antibiotics. No generally effective therapeutic agent that directly improves periodontal hard tissues has been developed.

[0024] Another major clinical problem in mineral metabolism is excessive loss or waste of phosphate (PO_4) out of the body system. Phosphate plays variety of important roles in all living creatures. In vertebrates, phosphate is a major component of their skeleton.

In all animals, phosphate is an essential component to build polynucleotide chains and cell membranes; phosphorylation and dephosphorylation of sugars and nucleotides are the most essential reactions in energy generation and consumption; and phosphorylation and dephosphorylation of proteins, sugars, and lipids are indispensable reactions for signal transduction in the cells. Therefore, a shortage of phosphate could even result in death.

- [0025] In mammals, phosphate concentration in the body fluid is controlled within a range that allows all normal biological functions in the body. The kidney is the most important organ for controlling phosphate levels in the body. Glomeruli in the kidney filter phosphate constantly to the urine, and proximal tubules usually reabsorb approximately 80% of this filtered phosphate. If this reabsorbing function is damaged, excessive phosphate is lost into the urine, resulting in various clinical problems.
- [0026] For instance, it is well known that the majority of kidney transplant patients experience excessive renal phosphate leakage, because the transplanted kidneys only marginally reabsorb the urinary phosphate to the circulation. The reasons for this poor reabsorbing activity on the part of transplanted kidneys are unknown. It frequently causes the patients malnutrition and secondary osteoporosis. This problem cannot be treated by a simple exogenous supplementation of phosphate. Similar renal phosphate leakage with unknown pathology is often observed in pediatric medicine, with outcomes such as malnutrition or growth retardation.
- [0027] Health problems associated with circulating phosphate shortage is not limited to humans. Milking cows sometimes suffer from hypophosphatemia (too low phosphate in the blood) by overproduction of the milk. It not only deteriorates the nutritional quality of the milk but also often make the cows useless for milk production. It is as relatively common problem in dairy farms.
- [0028] Clearly, there is a significant demand for a therapeutic agent that promotes regeneration of alveolar bone and/or teeth, increases the number and activity of odontoblasts/osteoblasts that help form dental tissues, and reduces renal phosphate secretion.

SUMMARY OF THE INVENTION

- [0029] A class of compounds is disclosed which are useful in treating or preventing a condition associated with skeletal loss or weakness and/or which reduce renal phosphate excretion. The compounds are peptides or analogs thereof which comprise between 10 and 50 monomer (e.g. amino acids) units. The amino acid sequence comprises one or more of the following motifs: an integrin binding motif sequence; a glycosaminoglycan binding motif; and a calcium-binding motif. The amino acids may be in the D- or L-conformation. The remaining monomer units (the sequence other than the aforementioned motifs) in the compound may be amino acid analogs. Where the motif is an integrin binding motif, the remaining monomer units are preferably naturally occurring amino acids having a sequence which are substantially the same as an amino acid sequence contiguous with the RGD sequence in the naturally occurring protein, matrix extracellular phosphoglycoprotein (Rowe et. al., *Genomics* (2000) 67:56-68).
- [0030] An aspect of the invention is a set of peptides and/or peptide analogs.
- [0031] A feature of the invention is that a compound of the invention comprises one or more of the following motifs: an integrin binding motif sequence; a glycosaminoglycan-binding motif; and a calcium-binding motif. The amino acids may be in the D- or L-conformation.
- [0032] An advantage of the invention is that a compound of the invention enhances skeletal growth.
- [0033] Another advantage of the invention is that a compound of the invention enhances the number of osteoblast and possibly odontoblast cells on the surface of new skeletal or tooth growth.
- [0034] Another advantage of the invention is that a compound of the invention reduces phosphate (Pi) loss from the body, as indicated by reduced urinary Pi leakage.
- [0035] Another aspect of the invention is to provide a formulation for therapeutic use which comprises a sufficient concentration of a compound of the invention and can be administered to the pulp of teeth, the space between the root of teeth and gum, or alveolar bone to prevent the damage on teeth and/or alveolar bone or regenerate the hard tissue in the damaged teeth and/or alveolar bone.

- [0036] Another aspect of the invention is to provide toothpaste which comprises a sufficient concentration of a compound of the invention to enhance tooth and/or alveolar bone growth on areas where deterioration has occurred, or to prevent such deterioration.
- [0037] Yet another aspect of the invention is to provide a mouthwash which comprises a sufficient concentration of a compound of the invention to enhance tooth and/or alveolar bone growth on areas where deterioration has occurred, or to prevent such deterioration.
- [0038] Still another aspect of the invention is a dental floss having coated thereon and/or embedded therein a compound of the invention in an amount such that repeated application to teeth and/or alveolar bone results in enhanced tooth and/or alveolar bone growth on areas where deterioration has occurred, or to prevent such deterioration.
- [0039] A further aspect of the invention is a small adhesive patch for application on gum tissue of an individual, the patch comprising a therapeutically effective amount of a compound of the invention. The compound is slowly released from the patch into the gum, so that the released compound penetrates into the root of the teeth as well as the alveolar and/or jaw bones to prevent loss of such bones and/or to regenerate such bones.
- [0040] An object of the invention is to provide a method of treating or preventing skeletal bone or dental bone loss by the administration/application of any formulation/composition of the invention.
- [0041] These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the subject invention, as more fully described below.

BRIEF DESCRIPTIONS OF THE DRAWINGS

- [0042] Figure 1 is a graph showing the relationship between bone mass and age in humans.
- [0043] Figure 2 is a schematic drawing of a matrix extracellular phosphoglycoprotein wherein the area designated as "A" includes sequences which match peptides of the present invention and the area designated as "B" is a highly homologous motif to a group of bone-tooth matrix phosphoglycoproteins such as osteopontin (OPN), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and bone sialoprotein II (IBSP).

- [0044] Figures 3A, 3B, 3C, and 3D are actual photographs of bone cross-sections (from a seven day mouse calvaria organ culture study) showing the effects of a control (Figure 3A), fibroblast growth factor-1 (FGF-1) (Figure 3B), and two peptides of the invention designated D-00004 and D-00006 (Figure 3C and 3D, respectively).
- [0045] Figure 4 is a graph comparing the effects of different compounds on calvaria.
- [0046] Figure 5 is a graph showing the in vivo effects of D-00006.
- [0047] Figure 6 is a graph showing the effect of D-00006 on urinary phosphate leakage.

DETAILED DESCRIPTION OF THE INVENTION

- [0048] Before the peptides, analogs, formulations, and methodology of the present invention are described, it is to be understood that this invention is not limited to any particular embodiment described, as such may, of course, vary. It is also to be understood that the terminology used herein is with the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.
- [0049] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.
- [0050] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein

656142625-00025

are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

- [0051] It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a peptide" includes a plurality of such peptides and reference to "the method" includes reference to one or more methods and equivalents thereof known to those skilled in the art, and so forth.
- [0052] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DEFINITIONS

- [0053] The terms "peptide" and "peptidic compound" are used interchangeably herein to refer to a polymeric form of amino acids of from about 10 to about 50 amino acids, which can comprise coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, L- or D- amino acids, peptides having modified peptide backbones, and peptides comprising amino acid analogs. The peptidic compounds may be polymers of: (a) naturally occurring amino acid residues; (b) non-naturally occurring amino acid residues, e.g. N-substituted glycines, amino acid substitutes, etc.; or (c) both naturally occurring and non-naturally occurring amino acid residues/ substitutes. In other words, the subject peptidic compounds may be peptides or peptoids. Peptoid compounds and methods for their preparation are described in WO 91/19735, the disclosure of which is herein incorporated by reference.
- [0054] The terms "treat", "treating", "treatment" and the like are used interchangeably herein and mean obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of partially or completely curing a disease and/or adverse effect attributed to the disease such as enhancing the effect of vitamin D. "Treating" as used herein covers treating a disease in a vertebrate and particularly a mammal and most particularly a human, and includes:

- (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it;
- (b) inhibiting the disease, i.e. arresting its development; or
- (c) relieving the disease, i.e. causing regression of the disease.

[0055] The invention is particularly directed towards peptides which make it possible to treat patient's which have experienced bone loss or which would be expected to experience bone loss and thus is particularly directed towards preventing, inhibiting, or relieving the effects of bone loss. A subject is "treated" provided the subject experiences a therapeutically detectable and beneficial effect which may be measured based on a variety of different criteria including increased bone growth, increased bone strength or other characteristics generally understood by those skilled in the art to be desirable with respect to the treatment of diseases related to bone.

[0056] The term "antibody" is meant an immunoglobulin protein capable of binding an antigen. The term "antibody" as used herein is intended to include antibody fragments (e.g. F(ab')₂, Fab', and Fab) capable of binding an antigen or antigenic fragment of interest.

[0057] The term "binds specifically" is meant high avidity and/or high affinity binding of an antibody to a specific peptide -- specifically a peptide of the invention. Antibody binding to its specific target epitope is stronger than the binding of the antibody to other epitopes on the peptide or to other epitopes on other peptides. Antibodies which bind specifically to a peptide of interest may be capable of binding to other peptides at a weak, yet detectable level (e.g. 10% or less of the binding shown to the peptide of interest). Such weak binding or background binding, is readily discernable from the specific antibody binding to the peptide of interest, e.g. by the use of appropriate controls.

[0058] The term "skeletal loss" refers to any situation in which skeletal mass, substance or matrix or any component of the skeleton, such as calcium and phosphate, is decreased or the bone is weakened such as in terms of its ability to resist being broken.

[0059] The term "skeleton" includes both bone and teeth. In the same manner, the term "skeletal" means both bone and teeth.

[0060] The term "osteoporosis" is intended to refer to any condition involving bone loss, i.e. involving a reduction in the amount of bone mass or substance resulting from any

5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
4410
4411
4412
4413
4414
4415
4416
4417
4418
4419
4420
4421
4422
4423
4424
4425
4426
4427
4428
4429
4430
4431
4432
4433
4434
4435
4436
4437
4438
4439
4440
4441
4442
4443
4444
4445
4446
4447
4448
4449
44410
44411
44412
44413
44414
44415
44416
44417
44418
44419
44420
44421
44422
44423
44424
44425
44426
44427
44428
44429
44430
44431
44432
44433
44434
44435
44436
44437
44438
44439
44440
44441
44442
44443
44444
44445
44446
44447
44448
44449
444410
444411
444412
444413
444414
444415
444416
444417
444418
444419
444420
444421
444422
444423
444424
444425
444426
444427
444428
444429
444430
444431
444432
444433
444434
444435
444436
444437
444438
444439
444440
444441
444442
444443
444444
444445
444446
444447
444448
444449
4444410
4444411
4444412
4444413
4444414
4444415
4444416
4444417
4444418
4444419
4444420
4444421
4444422
4444423
4444424
4444425
4444426
4444427
4444428
4444429
4444430
4444431
4444432
4444433
4444434
4444435
4444436
4444437
4444438
4444439
4444440
4444441
4444442
4444443
4444444
4444445
4444446
4444447
4444448
4444449
44444410
44444411
44444412
44444413
44444414
44444415
44444416
44444417
44444418
44444419
44444420
44444421
44444422
44444423
44444424
44444425
44444426
44444427
44444428
44444429
44444430
44444431
44444432
44444433
44444434
44444435
44444436
44444437
44444438
44444439
44444440
44444441
44444442
44444443
44444444
44444445
44444446
44444447
44444448
44444449
444444410
444444411
444444412
444444413
444444414
444444415
444444416
444444417
444444418
444444419
444444420
444444421
444444422
444444423
444444424
444444425
444444426
444444427
444444428
444444429
444444430
444444431
444444432
444444433
444444434
444444435
444444436
444444437
444444438
444444439
444444440
444444441
444444442
444444443
444444444
444444445
444444446
444444447
444444448
444444449
4444444410
4444444411
4444444412
4444444413
4444444414
4444444415
4444444416
4444444417
4444444418
4444444419
4444444420
4444444421
4444444422
4444444423
4444444424
4444444425
4444444426
4444444427
4444444428
4444444429
4444444430
4444444431
4444444432
4444444433
4444444434
4444444435
4444444436
4444444437
4444444438
4444444439
4444444440
4444444441
4444444442
4444444443
4444444444
4444444445
4444444446
4444444447
4444444448
4444444449
44444444410
44444444411
44444444412
44444444413
44444444414
44444444415
44444444416
44444444417
44444444418
44444444419
44444444420
44444444421
44444444422
44444444423
44444444424
44444444425
44444444426
44444444427
44444444428
44444444429
44444444430
44444444431
44444444432
44444444433
44444444434
44444444435
44444444436
44444444437
44444444438
44444444439
44444444440
44444444441
44444444442
44444444443
44444444444
44444444445
44444444446
44444444447
44444444448
44444444449
444444444410
444444444411
444444444412
444444444413
444444444414
444444444415
444444444416
444444444417
444444444418
444444444419
444444444420
444444444421
444444444422
444444444423
444444444424
444444444425
444444444426
444444444427
444444444428
444444444429
444444444430
444444444431
444444444432
444444444433
444444444434
444444444435
444444444436
444444444437
444444444438
444444444439
444444444440
444444444441
444444444442
444444444443
444444444444
444444444445
444444444446
444444444447
444444444448
444444444449
4444444444410
4444444444411
4444444444412
4444444444413
4444444444414
4444444444415
4444444444416
4444444444417
4444444444418
4444444444419
4444444444420
4444444444421
4444444444422
4444444444423
4444444444424
4444444444425
4444444444426
4444444444427
4444444444428
4444444444429
4444444444430
4444444444431
4444444444432
4444444444433
4444444444434
4444444444435
4444444444436
4444444444437
4444444444438
4444444444439
4444444444440
4444444444441
4444444444442
4444444444443
4444444444444
4444444444445
4444444444446
4444444444447
4444444444448
4444444444449
44444444444410
44444444444411
44444444444412
44444444444413
44444444444414
44444444444415
44444444444416
44444444444417
44444444444418
44444444444419
44444444444420
44444444444421
44444444444422
44444444444423
44444444444424
44444444444425
44444444444426
44444444444427
44444444444428
44444444444429
44444444444430
44444444444431
44444444444432
44444444444433
44444444444434
44444444444435
44444444444436
44444444444437
44444444444438
44444444444439
44444444444440
44444444444441
44444444444442
44444444444443
44444444444444
44444444444445
44444444444446
44444444444447
44444444444448
44444444444449
444444444444410
444444444444411
444444444444412
444444444444413
444444444444414
444444444444415
444444444444416
444444444444417
444444444444418
444444444444419
444444444444420
444444444444421
444444444444422
444444444444423
444444444444424
444444444444425
444444444444426
444444444444427
444444444444428
444444444444429
444444444444430
444444444444431
444444444444432
444444444444433
444444444444434
444444444444435
444444444444436
444444444444437
444444444444438
444444444444439
444444444444440
444444444444441
444444444444442
444444444444443
444444444444444
444444444444445
444444444444446
444444444444447
444444444444448
444444444444449
4444444444444410
4444444444444411
4444444444444412
4444444444444413
4444444444444414
4444444444444415
4444444444444416
4444444444444417
4444444444444418
4444444444444419
4444444444444420
4444444444444421
4444444444444422
4444444444444423
4444444444444424
4444444444444425
4444444444444426
4444444444444427
4444444444444428
4444444444444429
4444444444444430
4444444444444431
4444444444444432
4444444444444433
4444444444444434
4444444444444435
4444444444444436
4444444444444437
4444444444444438
4444444444444439
4444444444444440
4444444444444441
4444444444444442
4444444444444443
4444444444444444
4444444444444445
4444444444444446
4444444444444447
4444444444444448
4444444444444449
44444444444444410
44444444444444411
44444444444444412
44444444444444413
44444444444444414
44444444444444415
44444444444444416
44444444444444417
44444444444444418
44444444444444419
44444444444444420
44444444444444421
44444444444444422
44444444444444423
44444444444444424
44444444444444425
44444444444444426
44444444444444427
44444444444444428
44444444444444429
44444444444444430
44444444444444431
44444444444444432
44444444444444433
44444444444444434
44444444444444435
44444444444444436
44444444444444437
44444444444444438
44444444444444439
44444444444444440
44444444444444441
44444444444444442
44444444444444443
44444444444444444
44444444444444445
44444444444444446
44444444444444447
44444444444444448
44444444444444449
444444444444444410
444444444444444411
444444444444444412
444444444444444413
444444444444444414
444444444444444415
444444444444444416
444444444444444417
444444444444444418
444444444444444419
444444444444444420
444444444444444421
444444444444444422
444444444444444423
444444444444444424
444444444444444425
444444444444444426
444444444444444427
444444444444444428
444444444444444429
444444444444444430
444444444444444431
444444444444444432
444444444444444433
444444444444444434
444444444444444435
444444444444444436
444444444444444437
444444444444444438
444444444444444439
444444444444444440
444444444444444441
444444444444444442
444444444444444443
444444444444444444
444444444444444445
444444444444444446
444444444444444447
444444444444444448
444444444444444449
4444444444444444410
4444444444444444411
4444444444444444412
4444444444444444413
4444444444444444414
4444444444444444415
4444444444444444416
4444444444444444417
4444444444444444418
4444444444444444419
4444444444444444420
4444444444444444421
4444444444444444422
4444444444444444423
4444444444444444424
4444444444444444425
4444444444444444426
4444444444444444427
4444444444444444428
4444444444444444429
4444444444444444430
4444444444444444431
4444444444444444432
4444444444444444433
4444444444444444434
4444444444444444435
4444444444444444436
4444444444444444437
4444444444444444438
4444444444444444

cause. The term particularly results in a bone loss resulting from demineralization of the bone, post menopausal or peri-menopausal estrogen decrease or nerve damage.

[0061] The terms "subject," "individual," "patient," and "host" are used interchangeably herein and refer to any vertebrate, particularly any mammal and most particularly including human subjects, farm animals, and mammalian pets.

PEPTIDIC COMPOUNDS

[0062] A peptidic compound of the invention is a peptide comprising from 10 to 50 amino acids. The amino acids are preferably one of the twenty naturally occurring L-amino acids. However, D-amino acids may be present as may amino acid analogs. A peptide of the invention will comprise one or more of the following amino acid sequence motifs: an integrin binding motif such as RGD sequence; a glycosaminoglycan binding motif; and a calcium binding motif. Individual amino acids may be present in the peptides in either the L or the D isoform, but preferably in the L form. A peptide of the invention can be amidated or non-amidated on its C-terminus, or carboxylated or non-carboxylated on its N-terminus. The peptide of the invention may or may not contain a glycosaminoglycan binding motif such as SGD (SEQ ID NO:41) sequence in L- or D-isomer form. A compound of the invention is still further characterized by biological activity i.e. it enhances skeletal growth as well as the growth or recruiting of osteoblast or odontoblast cells on surface of the new skeletal growth.

[0063] A peptidic compound of the invention exhibit one or more of the following properties when administered in an effective amount to an individual: (1) reduce bone loss; (2) increase bone mass; (3) increase bone strength; (4) reduce renal excretion of phosphate; and (5) reduce loss of phosphate from an individual.

[0064] Specific examples of peptides of the invention comprise seven to forty-seven amino acids on either side of the RGD sequence of the naturally occurring sequence of matrix extracellular phosphoglycoprotein. Thus, examples of peptides of the invention comprising sequences taken from the following sequence and including the RGD sequence shown in bold:

DSQAQKSPVSKSTHRIQHNIDYLKHLSKVKKIPSDFEGSGYTDLQERGDNDISPFSGDG
QPKDIPGKGEATGPDLEGKDIQTGFAGPSEAESTHL (SEQ ID NO:1)

[0065] Specific examples of peptides of the invention which comprise the RGD sequence as the terminal sequence include the following:

AQKSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSDFEGSGYTDLQERGD (SEQ ID NO:2)
RGDAQKSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSDFEGSGYTDLQE (SEQ ID NO:3)
DSQAQKSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSDFEGSGYTDRGD (SEQ ID NO:4)
RGDSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSDFEGSGYTDLQE (SEQ ID NO:5)
DSQAQKSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSDFEGSGRGRD (SEQ ID NO:6)
RGDT HRIQHNIDYLKHL SKVKKIPSDFEGSGYTDLQE (SEQ ID NO:7)
DSQAQKSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSDFERGD (SEQ ID NO:8)
RGDL KHL SKVKKIPSDFEGSGYTDLQE (SEQ ID NO:9)
DSQAQKSPVSKSKSTHRIQHNIDYLKHL SKVKKIPSRGD (SEQ ID NO:10)
RGDSL KHL SKVKKIPSDFEGSGYTDLQE (SEQ ID NO:11)
DSQAQKSPVSKSKSTHRIQHNIDYLKHL SKRGD (SEQ ID NO:12)
RGDVK KIPSDFEGSGYTDLQE (SEQ ID NO:13)
DSQAQKSPVSKSKSTHRIQHNIDYLKRGD (SEQ ID NO:14)
RGDIP PSDFEGSGYTDLQE (SEQ ID NO:15)
DSQAQKSPVSKSKSTHRIQHNIDRGD (SEQ ID NO:16)
RGDD FEGSGYTDLQE (SEQ ID NO:17)
DSQAQKSPVSKSKSTHRRGD (SEQ ID NO:18)
RGDG SGYTDLQE (SEQ ID NO:19)
DSQAQKSPVKRGD (SEQ ID NO:20)
RGDGYTDLQE (SEQ ID NO:21)
DSQAQKSRGD (SEQ ID NO:22)
RGDN DISPFSGDGQPFKDIPGKGEATGPDLEGKDIQTGFA (SEQ ID NO:23)

[0066] Specific examples of the peptides of the invention which comprise the RGD internally include the following:

NDI **RGDSPFSGDGQPFKDIPGKGEATGPDLEGKDIQTGFA** (SEQ ID NO:24)
NDISPF **RGDSGDGQPFKDIPGKGEATGPDLEGKDI** (SEQ ID NO:25)

NDISPFGD **RGDGQPFKDI**PGKGEATGPDL (SEQ ID NO:26)
 FSGDGQPFKDI~~PGKGEATGP~~LEGKDIQTGFAGPSEAES **RGDTHL** (SEQ ID NO:27)
 IPGKGEATGP~~DLEGKDI~~QTGFAGPSE **RGDAESTHL** (SEQ ID NO:28)
 EATGP~~DLEGKDI~~QTGFAG **RGDPSEAESTHL** (SEQ ID NO:29)
 NDISPFGDGGQPKD **RGDIPGKGEATGP**LEGK (SEQ ID NO:30)
 GKGEATGP~~DLEGKDI~~ QTGFAGPSEAESTHL (SEQ ID NO:31)
 FSGDGQPFKDI~~PGKGEATG~~ RGDPDLEGKDIQTGFAGPSEA (SEQ ID NO:32)
 DGQPFKDI~~PGKGEATG~~ RGDPDLEGKDIQTGF (SEQ ID NO:33)
 PFKDIPGKGEATG **RGD**PDLEGKDIQ (SEQ ID NO:34)
 DIPGKGEATG **RGD**PDLEGKDIQTGFAGP (SEQ ID NO:35)
 DGQPFKDI~~PGKGEATG~~ RGDPDLEGKDIQTGF (SEQ ID NO:36)
 GKGEATG **RGD**PDLEGKDIQTGFAGPSEA (SEQ ID NO:37)
 EATG **RGD**PDLEGKDIQTGF (SEQ ID NO:38)
 EATG **RGD**PDLEGK (SEQ ID NO:39)
 EATG **RGD**PDL (SEQ ID NO:40)

[0067] In some embodiments, a peptide of the invention comprises a glycosaminoglycan-binding motif. A glycosaminoglycan binding motif has the consensus sequence SGXG (SEQ ID NO:50), wherein X is any amino acid. In some embodiments, a glycosaminoglycan binding motif has the sequence SGDG (SEQ ID NO:41).

[0068] In other embodiments, a peptide of the invention comprises a calcium binding motif. In some embodiments, a calcium binding motif has the sequence DNDISPFGDQQ (SEQ ID NO:42). Also included in the term “calcium binding motif” are amino acid sequences that differ from SEQ ID NO:42 by one, two, three, four, five, six, seven, or eight amino acids. Of particular interest in many embodiments are motifs that conserve amino acids 1, 3, 5, 7, 9, and 12 of SEQ ID NO:42. Thus, in some embodiments, a peptide of the invention comprises, as a calcium-binding motif, the sequence DXDXSXFXGXXQ (SEQ ID NO:43), wherein X is any amino acid or amino acid analog.

[0069] In other embodiments, a calcium binding motif has the sequence DX₁X₂X₃X₄X₅X₆X₇X₈X₉X₁₀X₁₁X₁₂, wherein:

X_1 is any amino acid;
 X_2 is D, N, or S;
 X_3 is I, L, V, F, Y, or W;
 X_4 is D, E, N, S, T, or G;
 X_5 is D, N, Q, G, H, R, or K;
 X_6 is G or P;
 X_7 is L, I, V, M, C;
 X_8 is D, E, N, Q, S, T, A, G, or C;
each of X_9 and X_{10} is independently any amino acid;
 X_{11} is D or E; and
 X_{12} is L, I, V, M, F, Y, or W.

[0070] In other embodiments, a calcium binding motif has the sequence

$X_1X_2X_3X_4C(X_5)_nC(X_6)_mCX_7X_8X_9X_{10}X_{11}X_{12}X_{13}X_{14}C$, wherein
each of X_1 , X_3 , and X_4 is independently D, E, Q, or N;
each of X_2 , X_5 , X_6 , X_7 , X_9 , X_{10} , X_{11} , X_{12} , and X_{14} is independently any amino acid;
n is 3-14;
m is 3-7;
 X_8 is D or N; and
 X_{13} is F or Y.

[0071] In other embodiments, a calcium binding motif has the sequence

$X_1X_2X_3X_4X_5DX_6X_7X_8X_9X_{10}X_{12}X_{13}X_{14}X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}$, wherein
each of X_1 and X_2 is independently L, I, V, M, F, Y, or W;
each of X_3 , X_4 , X_6 , X_7 , X_8 , X_{10} , X_{11} , X_{12} , X_{15} , X_{18} , and X_{19} is independently any amino acid;

X_5 is L or K;
 X_9 is D or N;
 X_{13} is D, N, S, or G;
 X_{14} is F or Y;
 X_{16} is E or S;
 X_{17} is F, Y, V, or C;

X₂₀ is L, I, V, M, F, or S;
and X₂₁ is L, I, V, M, or F.

[0072] In other embodiments, a calcium binding motif has the sequence DX₁X₂X₃X₄X₅X₆GX₇DX₈X₉X₁₀GGX₁₁X₁₂X₁₃D, wherein each of X₁, X₃, X₄, X₅, X₆, X₇, X₈, X₁₀, X₁₁, X₁₂, and X₁₃ is independently any amino acid; and
each of X₂ and X₉ is independently L or I.

[0073] Calcium binding motifs are known in the art and have been described amply. See, for example, Springer et al. (2000) *Cell* 102:275-277; Kawasaki and Kretsinger (1995) *Protein Prof.* 2:305-490; Moncrief et al. (1990) *J. Mol. Evol.* 30:522-562; Chauvaux et al. (1990) *Biochem. J.* 265:261-265; Bairoch and Cox (1990) *FEBS Lett.* 269:454-456; Davis (1990) *New Biol.* 2:410-419; Schaefer et al. (1995) *Genomics* 25:638-643; and Economou et al. (1990) *EMBO J.* 9:349-354. Any known calcium binding motif can be included in a peptidic compound of the invention.

[0074] A peptide of the invention may comprise one or more of an integrin binding motif, a glycosaminoglycan binding motif, and a calcium binding motif. The motifs may be present in the peptide in any order relative to one another. The motifs may be separated from one another by one, two, three, four, five, six, seven, eight, nine, or ten amino acids, or more. Furthermore, a motif may overlap with one or more other motifs. As one non-limiting example, a peptide having the sequence TDLQERGDNDISPFGSGDGQPKD (SEQ ID NO:49) comprises all three motifs, which overlap with one another.

[0075] All or any of the amino acids in the above sequences may be in the D- or L-conformation and may be substituted with equivalent analogs. The preferred embodiments comprise naturally occurring amino acids in the L-conformation.

[0076] All or any of the above sequences may be amidated, non-amidated, or otherwise modified on their C-terminus, or carboxylated, non-carboxylated, or otherwise modified on their N-terminus.

[0077] In addition, multimers of any of the foregoing peptides are provided. Multimers include dimers, trimers, tetramers, pentamers, hexamers, etc. Thus, a peptide of the

invention having a length of from about 10 to about 50 amino acids can be multimerized, optionally with an intervening linker, such that a subject peptide occurs in tandem arrays of two, three, four, five, six, or more copies. Furthermore, two or more different peptides of the invention can be multimerized with one another, forming “heteromultimers.” Thus, e.g., a multimer may comprise a first and a second peptide, linked together by peptide bonds, optionally with a linker molecule such as one to ten glycine residues.

[0078] Peptidic compounds of the invention can be obtained using any known method, including, e.g., solid phase peptide synthesis techniques, where such techniques are known to those of skill in the art. Methods for synthesizing peptides are well known in the art and have been amply described in numerous publications, including, e.g., “The Practice of Peptide Synthesis” M. Bodanszky and A. Bodanszky, eds. (1994) Springer-Verlag; and Jones, The Chemical Synthesis of Peptides (Clarendon Press, Oxford)(1994). Generally, in such methods a peptide is produced through the sequential additional of activated monomeric units to a solid phase bound growing peptide chain. Also of interest is the use of submonomers in solid phase synthesis, as described in WO 94/06451, the disclosure of which is herein incorporated by reference.

[0079] Instead of solid phase synthesis, the subject peptidic compounds of the subject invention may be prepared through expression of an expression system comprising a polynucleotide encoding the peptidic compound. Any convenient methodology may be employed, where methodologies that may be employed typically include preparation of a nucleic acid molecule comprising a nucleotide sequence encoding the subject peptide, introduction of the encoding region into a vector for expression, transformation of a host cell with the vector, and expression and recovery of the product. Protocols for accomplishing each of the above steps are well known in art. See Sambrook, Fritsch & Maniatis, Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Press, Inc.)(1989).

[0080] Matrix extracellular phosphoglycoprotein was cloned and characterized from a human tumor that caused osteomalacia in the patients. This extremely rare type of tumor called Oncogenic Hypophosphatemic Osteomalacia (OHO) tumor has been known to cause renal phosphate leak, hypophosphatemia (low serum phosphate levels), low serum calcitriol (1,25-vitamin D3), and abnormalities in skeletal mineralization (Osteomalacia).

In the patients of OHO tumor, resection of the tumors results in remission of all of the above symptoms and it has been proposed that a circulating phosphaturic factor secreted from OHO tumor plays a role in osteomalacia. Matrix extracellular phosphoglycoprotein was proposed as a candidate of this phosphaturic factor (Rowe et al., *Genomics* (2000) 67:56-68).

- [0081] Phosphate plays a central role in many of the basic processes essential to the cell and the mineralization of skeleton. In particular, skeletal mineralization is dependent on the regulation of phosphate and calcium in the body and any disturbances in phosphate-calcium homeostasis can have severe repercussions on the integrity of bone. In the kidney, phosphate is lost passively into the glomerular filtrate and is actively reabsorbed via a sodium (Na^+) dependent phosphate cotransporter. In the intestine, phosphate is absorbed from foods. A sodium (Na^+) dependent phosphate cotransporter was found to be expressed in the intestine and recently cloned (Hilfiker, PNAS 95(24) (1998), 14564-14569). The liver, skin and kidney are involved in the conversion of vitamin D₃ to its active metabolite, calcitriol, which plays an active role in the maintenance of phosphate balance and skeletal mineralization.
- [0082] Vitamin D deficiency causes rickets in children and osteomalacia in adults. Both conditions are characterized by failure of calcification of osteoid, which is the matrix of skeleton.
- [0083] Thus, all of the humoral functions by matrix extracellular phosphoglycoprotein, namely, renal phosphate leak, hypophosphatemia (low serum phosphate levels), low serum calcitriol (1,25-vitamin D₃), are harmful to healthy skeletal formation.
- [0084] Matrix extracellular phosphoglycoprotein is a large polypeptide with 525 amino acid with short N-terminus signal sequence. Therefore, it is highly probable that this molecule is secreted from its producing cells into the body fluid and circulation. Out of its 525 amino acid sequence, a 23 amino acid motif on the C-terminus showed high similarities to a group of bone-tooth mineral matrix phosphoglycoproteins such as osteopontin (OPN), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), and bone sialoprotein II (IBSP). It has been proposed that these bone-tooth mineral matrix phosphoproteins may play important roles in skeletal mineralization.
- [0085] Notwithstanding the above observations about matrix extracellular phosphoglycoprotein, smaller peptide sequence containing integrin binding motif that is

located within the amino acid sequence and far from its C-terminus sequence with a high degree of similarity to other bone-tooth mineral matrix phosphoglycoproteins demonstrated a very potent skeletal formation activity and increased the number of osteoblasts on such skeletal formation surface. The potency of such activities was equivalent to fibroblast growth factor (FGF). It was surprising in that small motifs located within a larger protein which has destructive functions on the skeleton demonstrated potent bone formation activity, and that such motifs were located far from the sequence which showed homology to other known bone-tooth matrix proteins.

[0086] Another surprising fact was that potent skeletal formation motifs of the invention contained an integrin binding motif, in particular, RGD sequence. It has been reported that a synthetic peptide containing the RGD sequence inhibited bone formation and resorption in a mineralizing organ culture system of fetal rat skeleton (Gronowicz et. al. Journal of Bone and Mineral Research 9(2):193-201 (1994)), that is a very similar experimental method used to test the subject of the present invention.

[0087] Further, the skeletal formation activity provided by the small peptides of the invention was as potent as that of an intact growth factor such as FGF.

THERAPEUTIC METHODS

[0088] The invention provides methods for reducing skeletal bone loss, methods for reducing renal phosphate leakage, methods for increasing bone mass, methods for increasing bone strength, and methods for reducing Pi excretion, comprising administering a peptidic compound of the invention. Typically, a peptidic compound of the invention is formulated with a pharmaceutically acceptable excipient for delivery to an individual in need thereof.

[0089] As used herein, an “effective amount” of a peptidic compound of the invention is an amount that reduces bone loss, and/or increases bone strength, and/or increases bone mass, and/or reduces phosphate loss, and/or reduces renal phosphate excretion by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, or at least about 60%, or more, when compared to a suitable control. Suitable controls are, in the case of experimental animals, an animal not treated with the peptide, e.g., treated with vehicle, or treated with an irrelevant peptide; and in the case of

human subjects, a human subject treated with a placebo, or a human subject before treatment with a peptide of the invention.

[0090] In some embodiments, an effective amount of a peptidic compound of the invention is an amount that reduces renal phosphate excretion, and therefore reduces phosphate loss from an individual, by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, or at least about 60%, or more, when compared to a suitable control.

[0091] Whether a given peptide reduces bone loss, and/or increases bone strength, and/or increases bone mass, and/or reduces phosphate loss, and/or reduces renal phosphate excretion in an individual can be determined using any known assay to measure any known parameter associated with any one or more of reduced bone loss, increased bone strength, increased bone mass, reduces phosphate loss, and reduced renal phosphate excretion, including, but not limited to, serum and urinary phosphorus levels (e.g., using a colorimetric assay); serum and urinary calcium levels (e.g., using a colorimetric assay); serum and urinary creatinine levels; bone turnover marker levels (e.g., deoxypyridinoline and osteocalcin); bone density (e.g., by *in vivo* bone densitometry); bone mechanical testing (e.g., lumbar vertebrae compression test; femoral shaft three point bending test; and the like); and the like. Such methods are standard in the art.

[0092] Individuals suitable for treatment with the methods of the invention are individuals having believed to be at risk for, bone loss, or a disorder caused by bone loss, or a disorder whose sequelae include bone loss, including, but not limited to, dental caries, osteoporosis, Paget's disease, renal phosphate leakage, renal osteodystrophy, osteomalacia, osteodystrophy resulting from other causes, osteolysis mediated by cancer, fractures, and hyperparathyroidism. Such individuals include older individuals, post-menopausal women, kidney transplant recipients, and individuals having, or being at risk for, any of the aforementioned disorders.

Routes of administration

[0093] Peptides of the invention are administered to an individual using any available method and route suitable for drug delivery, including *in vivo* and *ex vivo* methods, as well as systemic and localized routes of administration.

- [0094] Conventional and pharmaceutically acceptable routes of administration include intranasal, intramuscular, intratracheal, subcutaneous, intradermal, topical application, intravenous, rectal, nasal, oral and other parenteral routes of administration. Routes of administration may be combined, if desired, or adjusted depending upon the immunomodulatory nucleic acid molecule and/or the desired effect on the immune response. Peptides of the invention can be administered in a single dose or in multiple doses.
- [0095] Peptides of the invention can be administered to a subject using any available conventional methods and routes suitable for delivery of conventional drugs, including systemic or localized routes. In general, routes of administration contemplated by the invention include, but are not necessarily limited to, enteral, parenteral, or inhalational routes.
- [0096] Parenteral routes of administration other than inhalation administration include, but are not necessarily limited to, topical, transdermal, subcutaneous, intramuscular, intraorbital, intracapsular, intraspinal, intrasternal, and intravenous routes, *i.e.*, any route of administration other than through the alimentary canal. Parenteral administration can be carried to effect systemic or local delivery of peptides of the invention. Where systemic delivery is desired, administration typically involves invasive or systemically absorbed topical or mucosal administration of pharmaceutical preparations.
- [0097] Peptides of the invention can also be delivered to the subject by enteral administration. Enteral routes of administration include, but are not necessarily limited to, oral and rectal (*e.g.*, using a suppository) delivery.
- [0098] Methods of administration of a peptide of the invention through the skin or mucosa include, but are not necessarily limited to, topical application of a suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration. Also contemplated for delivery of a peptide of the invention is a patch containing therein a peptide of the invention. A patch can be applied to the skin, or to other tissue, *e.g.*, gum tissue. Any known patch delivery system that is suitable for oral delivery system can be used. See, *e.g.*, U.S. Patent No. 6,146,655.
- [0099] Peptides of the invention can also be delivered to an individual by administering to the individual a nucleic acid molecule comprising a nucleotide sequence that encodes a peptide of the invention. The terms “polynucleotide” and “nucleic acid molecule” are

used interchangeably herein to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. For expression, an expression cassette may be employed. The expression vector will provide a transcriptional and translational initiation region, which may be inducible or constitutive, where the coding region is operably linked under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination region. These control regions may be native to a gene encoding the subject peptides, or may be derived from exogenous sources.

- [00100] Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences encoding heterologous proteins. A selectable marker operative in the expression host may be present. Expression vectors may be used for the production of fusion proteins, where the exogenous fusion peptide provides additional functionality, i.e. increased protein synthesis, stability, reactivity with defined antisera, an enzyme marker, e.g. β -galactosidase, etc.

[00101] Expression cassettes may be prepared comprising a transcription initiation region, the gene or fragment thereof, and a transcriptional termination region. Vectors include, but are not limited to, plasmids; cosmids; viral vectors; artificial chromosomes (YAC's, BAC's, etc.); mini-chromosomes; and the like. Vectors are amply described in numerous publications well known to those in the art, including, e.g., Short Protocols in Molecular Biology, (1999) F. Ausubel, et al., eds., Wiley & Sons.

[00102] Expression vectors may be used to introduce a nucleic acid molecule encoding a subject peptide into a cell of an individual. Such vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences. Transcription cassettes may be prepared comprising a transcription initiation region, the target gene or fragment thereof, and a transcriptional termination region. The transcription cassettes may be introduced into a variety of vectors, e.g. plasmid; retrovirus, e.g. lentivirus; adenovirus; and the like, where the vectors are able to transiently or stably be maintained in the cells, usually for a period of at least about one day, more usually for a period of at least about several days to several weeks.

[00103] An expression vector comprising a nucleotide sequence encoding a peptide of the invention may be introduced into tissues or host cells by any number of routes, including

viral infection, microinjection, or fusion of vesicles. Jet injection may also be used for intramuscular administration, as described by Furth *et al.* (1992), *Anal Biochem* 205:365-368. The expression vector may be coated onto gold microparticles, and delivered intradermally by a particle bombardment device, or "gene gun" as described in the literature (see, for example, Tang *et al.* (1992), *Nature* 356:152-154), where gold microprojectiles are coated with the expression vector, then bombarded into skin cells.

Dosages

- [00104] Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1 μ g, to about 1,000 μ g, to about 10,000 μ g, to about 25,000 μ g or about 50,000 μ g of a peptide of the invention. Peptides of the invention can be administered in a single dosage or several smaller dosages over time. Alternatively, a target dosage of a peptide can be considered to be about 0.1- 1000 μ M, about 1-500 μ M, or about 5-250 μ M in a sample of host blood drawn within the first 24-48 hours after administration of the peptide.
- [00105] The effect on bone loss, bone strength, phosphate excretion, or other parameter may be dose-dependent. Therefore, to increase potency by a magnitude of two, each single dose is doubled in concentration. Increased dosages may be needed to achieve the desired therapeutic goal. The invention thus contemplates administration of multiple doses to provide and maintain an effect on bone loss, bone strength, phosphate excretion, or other parameter. When multiple doses are administered, subsequent doses are administered within about 16 weeks, about 12 weeks, about 8 weeks, about 6 weeks, about 4 weeks, about 2 weeks, about 1 week, about 5 days, about 72 hours, about 48 hours, about 24 hours, about 12 hours, about 8 hours, about 4 hours, or about 2 hours or less of the previous dose.
- [00106] In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of peptides according to the invention.

Formulations

- [00107] In general, peptides are prepared in a pharmaceutically acceptable composition for delivery to a host. Pharmaceutically acceptable carriers preferred for use with the peptides of the invention may include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol,

polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/ aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. A composition comprising a peptide of the invention may also be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention. Also of interest are formulations for liposomal delivery, and formulations comprising microencapsulated peptides.

[00108] In general, the pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. In some embodiments, where delivery of a peptide of the invention is to oral tissues, a peptide of the invention may be formulated in a toothpaste, a mouthwash, or may be coated on or embedded in a dental floss. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions comprising the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents. Preservatives and other additives may also be present such as, for example, anti-pathogenic agents (e.g., antimicrobials, antibacterials, antivirals, antifungals, etc.), antioxidants, chelating agents, and inert gases and the like.

[00109] A peptidic compound of the invention can be administered with any other known agent that reduces bone loss. Thus, combination therapy is contemplated. Other agents that can be administered with a peptide of the invention include, but are not limited to, estrogen, calcitonin, vitamin D, fluoride, Ipriflavon, and bisphosphonate. A peptide of the invention can be administered simultaneously with (e.g., in admixture with, or in separate formulations) another agent that reduces bone loss; or can be administered within about 15 minutes, about 30 minutes, about 60 minutes, about 2 hours, about 5 hours, about 10 hours, about 12 hours, about 24 hours, about 36 hours, about 4 days, about 7 days, or more, of another agent that reduces bone loss. In addition, two or more

peptides of the invention can be administered simultaneously or within about 15 minutes, about 30 minutes, about 60 minutes, about 2 hours, about 5 hours, about 10 hours, about 12 hours, about 24 hours, about 36 hours, about 4 days, about 7 days, or more of each other.

EXAMPLES

[00110] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

EXAMPLE 1

Synthesis of D-00001, etc.

[00111] Six different peptides were manually synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy and prepared in the C-terminal amide form. The six peptides are as follows:

D-00001:	IPSDFEGSGYTDLQE (SEQ ID NO:44)
D-00002:	DFEGSGYTDLQ EGRGD (SEQ ID NO:45)
D-00003:	YTDLQ EGRGDNDISPF (SEQ ID NO:46)
D-00004:	ERGDNDISPFSGDGQ (SEQ ID NO:47)
D-00005:	NDISPFSGDGQPFKD (SEQ ID NO:48)
D-00006:	TDLQ EGRGDNDISPFSGDGQPFKD (SEQ ID NO:49)
(C-terminus amidated)	

[00112] Amino acid derivatives and resins were purchased from Bachem, Inc., Torrance, CA., and Novabiochem, La Jolla, CA. The respective amino acids were condensed manually in a stepwise manner using 4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)-

phenoxy resin. N-methyl pyrrolidone was used during the synthesis as a solvent. For condensation, diisopropylcarbodiimide/N-hydroxybenzotriazole was employed, and for deprotection of N^a-Fmoc groups, 20% piperidine in N-methyl pyrrolidone was employed. The following side chain protecting groups were used: Asn and Gln, trityl; Asp, Glu, Ser, and Thr, t-butyl; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; and Lys, t-butoxycarbonyl. Resulting protected peptide resins were deprotected and cleaved from the resin using a trifluoroacetic acid-thioanisole-*m*-cresol-ethanedithiol-H₂O (80:5:5:5:5, v/v) at 20°C for 2 h. Resulting crude peptides were precipitated and washed with ethyl ether then purified by reverse-phase high performance liquid chromatography (using Vydac 5C18 column and a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid). All peptides were obtained with 5-20% yield (from the starting resin). Purity of the peptides was confirmed by analytical high performance liquid chromatography. Identity of the peptides was confirmed by a Sciex API IIIE triple quadrupole ion spray mass spectrometer.

EXAMPLE 2

Fetal Mouse Calvarial Assay

Reagents

- [00113] FGF-1 was purchased from Peprotech Inc. (Rocky Hill, NJ). RGD-1, 2, 3, 4, 5 and 6 (referred to here as D-00001, D-00002, D-00003, D-00004, D-00005 and D-00006) were provided by Dr. Nomizu (Hokkaido University, Japan).

Mice

- [00114] Pregnant ICR mice were purchased from SLC Japan Co. Ltd. (Shizuoka, Japan).

Mouse calvarial organ culture

- [00115] Mouse calvarial organ culture was performed as described in Mundy G et al. *Science* 286: 1946-1949, 1999 and Traianedes K et al. *Endocrinology* 139: 3178-3184, 1998. The calvaria from 4-days-old mice were excised and cut in half along the sagittal suture. Each half of the calvaria was placed on a stainless steel grid in a 12-well tissue culture dish (Asahi Glass Techno Corp., Funabashi, Japan). Each well contained 1.5 ml

of BGj medium (Sigma, St. Louis, MO) supplemented with 0.1% bovine serum albumin (Sigma) and each compound. FGF-1 was used as a positive control as described by Mundy et al. The medium was changed at day 1 and 4, and the assay was terminated at day 7.

Histomorphometrical analysis

[00116] Calvaria was fixed with 10% neutral-buffered formalin, decalcified with 4.13% EDTA and embedded in paraffin. 4mm-thickness sections were made and stained with hematoxylin and eosin. New bone area was measured using Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

[00117] The six peptides of Example 1 were tested for their ability to enhance bone growth with the tests being carried out as described above in Example 2. The peptides which did not include the RGD sequence did not show positive results. The other four peptides showed positive results with the best results being obtained with the sequences

D-00004: ERGDNDISPFGDGQ (SEQ ID NO:47), and

D-00006: TDLQERGDNDISPFGDGQPKD (SEQ ID NO:49).

[00118] The best results are in Figure 3 (specifically Figure 3C and 3D). Data from these results are graphically shown in Figures 4.

EXAMPLE 3

In Vivo Bone Formation Study

Reagents

[00119] FGF-1 was purchased from Peprotech Inc. (Rocky Hill, NJ). RGD-6 (referred to here as D-00006) was synthesized by CS Bio (San Carlos, CA) under the instruction of the inventors.

D-00006: TDLQERGDNDISPFGDGQPKD (SEQ ID NO:49).

Mice

[00120] Four week old mice were purchased from SLC Japan Co. Ltd. (Shizuoka, Japan) and randomized to three groups (n=5).

Mouse calvaria growth assay

[00121] D-00006 (20 μ g/kg/day), FGF-1 (12.5 μ g/kg/day), or vehicle (saline) was subcutaneously injected into the soft tissues adjacent to the calvariae of the test animals. The daily amount of the samples were divided by two, respectively, and injected twice a day for five days. After 15 days of the last administration, the calvariae were excised and cut in half along the sagittal suture, and provided to the Histomorphometrical analysis.

Histomorphometrical analysis

[00122] Calvaria was fixed with 10% neutral-buffered formalin, decalcified with 4.13% EDTA and embedded in paraffin. 4mm-thickness sections were made and stained with hematoxylin and eosin. New bone area was measured using Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Results

[00123] The calvaria sections from the animals treated with FGF-1 showed a significant expansion of bone area as compared to the vehicle treated group. Those from the animals treated with D-00006 also demonstrated a significant expansion of bone area as compared to the vehicle treated group and the efficacy was equivalent to that of FGF-1. Data from these results are graphically shown in Figure 5.

EXAMPLE 4

Effect of D-00006 on renal phosphate excretion*Experimental design and treatments*

[00124] Forty 3-month old virgin female Sprague-Dawley rats (Harlan Sprague Dawley, Inc.) were acclimated for one week prior to beginning the experiments. Following the acclimatization period, the animal were randomized by initial body weight into treatment groups outlined in Table 1. Ovx: ovariectomized; LD: low dose; HD: high dose; Est: estradiol; D-00006 (TDLQERGDNDISPFSGDGQPKD; SEQ ID NO:49).

Table 1

Group No.	Description	Treatment	Dose-Level	# of Animals
1	Sham	Vehicle	-	8
2	Ovx	Vehicle	-	8
3	Ovx + LD	D-00006	20 µg/kg/day	8
4	Ovx + HD	D-00006	200 µg/kg/day	8
5	Ovx + Est	17 β-estradiol pellet implant and vehicle	10 µg/kg/day	8

[00125] One day prior to the initiation of treatments, the animals were anesthetized with a ketamin/xylazine anesthetic mixture, and animals in groups 2-5 were ovariectomized.

[00126] Urine was collected in metabolic cages on Day 41. Blood and urine samples were collected for chemistry and bone turnover markers assays at the end of the 41-day treatment period. Prior to urine and blood collections, the animals were placed in metabolic cages and deprived of food for an overnight fast period of 18 hours. Urine samples were collected and the volumes recorded. The urine samples were then centrifuges at approximately 3000 x g for 10 minutes in a refrigerated centrifuge. The samples were filtered to remove contaminating sediments.

Serum and urine chemistry

[00127] Total serum calcium and creatinine levels were the same in all treatment groups, except that the Ovx animals treated with estradiol showed a slight increase in serum calcium. There was a dose-dependent increase in serum phosphorus in the D-0006 treated groups. Total urine volumes, collected over an 18-hour period, were the same in all groups. Figure 6 shows the derived urine parameters.

[00128] The total amount of phosphorus in the 18-hour urine collections showed a significant decrease in D-00006 treated groups. As a result, D-00006 treated groups demonstrated lower phosphate clearance and a higher percentage of tubular reabsorption of phosphorus. D-00006 was clearly shown to be an agent that conserves phosphorus in the circulation.

[00129] While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various

changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

66 9964264626 66 99642600